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# POLY(TRIMETHYLENE CARBONATE) AND POLY(D,L-LACTIC ACID) MODIFY HUMAN DENDRITIC CELL RESPONSES TO STAPHYLOCOCCI BUT DO NOT AFFECT Th1 AND Th2 CELL DEVELOPMENT

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**Abstract**

Biomaterial-associated infections (BAIs) are frequent complications in the use of medical devices (biomaterials) correlated with considerable patient discomfort and high treatment costs. The presence of a biomaterial in the host causes derangement of local immune responses increasing susceptibility to infection. Dendritic cells (DCs) have an important role in directing the nature of immune responses by activating and controlling $CD4^+$ T helper (Th) cell responses. To assess the immunomodulatory effect of the combined presence of biomaterials and *Staphylococcus aureus* (*S. aureus*) or *Staphylococcus epidermidis* (*S. epidermidis*), DC-mediated T cell proliferation and Th1/Th2 cell development were measured using an *in vitro* human cell system. Poly(trimethylene carbonate) (PTMC) and poly(D,L-lactic acid) (PDLLA) modified the production of the DC pro-inflammatory cytokines TNF-α, IL-6 and IL-23 in response to *S. aureus* and *S. epidermidis*. However, this modified cytokine production did not cause differences in Th1/Th2 cell polarisation, showing a Th1 cell predominance. In the absence of staphylococci, neither of the biomaterials induced DC-mediated T cell proliferation or Th1/Th2 cell polarisation. Moreover, either in the absence or presence of the biomaterials, *S. aureus* was a more potent inducer of DC cytokine secretion, T cell proliferation and Th1 cell development than *S. epidermidis*. In conclusion, although PTMC and PDLLA modulated DC cytokine responses to staphylococci, this did not alter the resulting Th cell development. This result suggested that, in this human cell model, Th1/Th2 cell responses were mainly determined by the species of bacteria and that PTMC or PDLLA did not detectably influence these responses.

**Keywords**: Biomaterial-associated infection, *Staphylococcus aureus*, *Staphylococcus epidermidis*, dendritic cell, T cell, immune response.

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## Introduction

Bacterial infections are a frequent complication when using medical devices (biomaterials) and they are associated with considerable morbidity and mortality and high hospital costs. These so-called biomaterial-associated infections (BAIs) are most often caused by staphylococci, in particular by the more virulent

*Staphylococcus aureus* (*S. aureus*) and the more benign *Staphylococcus epidermidis* (*S. epidermidis*) (Gandelman *et al.*, 2007; Zimmerli *et al.*, 2004). *S. aureus* produces numerous virulence factors, such as toxins and enzymes, and causes more acute implant infections when compared to *S. epidermidis*, which causes more subacute or even chronic implant infections (Vuong and Otto, 2002). BAIs are generally difficult to treat because bacteria can colonise the implanted biomaterial by forming a biofilm. In biofilms, bacteria are often more resistant to antibiotics and host defence mechanisms, when compared to their planktonic counterparts (Donlan and Costerton, 2002; Stewart and Costerton, 2001). However, infecting bacteria not only reside in biofilms on the biomaterial surface, but also in tissue surrounding the implant and even inside phagocytic cells (Boelens *et al.*, 2000a; Broekhuizen *et al.*, 2008) and osteoblasts (Bosse *et al.*, 2005). In a mouse experimental BAI model, macrophages in the tissue surrounding a subcutaneously implanted poly(vinylpyrrolidone)-coated catheter of silicon elastomer or polyamide show intracellular persistence of *S. epidermidis*, whereas bacterial challenge of mice without an implant leads to efficient bacterial clearance (Boelens *et al.*, 2000a; Broekhuizen *et al.*, 2010). Impairment of innate immune cell functions in BAIs is not restricted to macrophages nor to one type of biomaterial. In addition to macrophages, polymorphonuclear neutrophils (PMNs), harvested from subcutaneously implanted Teflon™ tissue cages, have a decreased bactericidal activity against *S. aureus* (Zimmerli *et al.*, 1984; Zimmerli and Sendi, 2011). Moreover, monocytes show decreased expression of major histocompatibility complex II (MHC-II) molecules in presence of poly(ethylene terephthalate) colonised with *S. epidermidis* (Henke *et al.*, 1997), which is expected to reduce antigen presentation and ensuing T cell activation. It is apparent that the local condition of the tissue surrounding an infected biomaterial provides signals that may impair proper innate immune cell function.

Currently, most of the studies investigating staphylococci-induced host immune responses in BAIs focus on innate immunity (Zimmerli and Sendi, 2011), whereas activation and regulation of adaptive immunity receive relatively little attention. Although innate immune cells are important in the first line defence against bacteria, they often need help from adaptive $CD4^+$ T helper (Th) cells. Th cells boost the microbicidal functions of a variety of innate immune cells, such as macrophages. In human patients, plenty of T cells are found at sites of biomaterial infection (Dapunt *et al.*, 2014; Wagner *et al.*, 2006); however, their exact role in BAIs is not known. $CD4^+$ Th cell activation and regulation is orchestrated by dendritic cells (DCs). Therefore, to understand the role of Th cells in BAIs, it is critical to study activation and regulation of Th cells by DCs.

Inadequate Th cell activation may be responsible for compromised immune defences around infected biomaterials. Protective adaptive immunity against staphylococci in humans, even in the absence of biomaterials, is not completely understood. Th1 and Th17 cell responses are thought to be protective against *S. aureus* infection (Brown *et al.*, 2015; Milner *et al.*, 2008), but not much is known about protective human Th cell responses against *S. epidermidis* infection. To the best of our knowledge, human Th cell responses to staphylococci in the presence of a biomaterial have not been characterised. However, in mice, Th1 and Th17 cell responses appear to play a role in BAIs. Treatment of mice with the Th1 cell effector cytokine interferon gamma (IFN-$\gamma$) reduces susceptibility to *S. epidermidis* infection around subcutaneously implanted catheter segments (Boelens *et al.*, 2000b), presumably since IFN-$\gamma$ enhances the microbicidal activity of phagocytes (Kubica *et al.*, 2008; Smith *et al.*, 2010). This indicates that poor Th1 cell development in BAIs may be responsible for inefficient *S. epidermidis* clearance. On the other hand, Th1/Th17 cell responses seem ineffective in the clearance of *S. aureus* murine prosthetic-implant infections, whereas Th2 and regulatory T cell responses protect from the inflammation (Prabhakara *et al.*, 2011; Rochford *et al.*, 2016). These studies indicate that, in mice, the combination of staphylococci and a biomaterial can influence Th cell responses in favour of staphylococcal infection. In this respect, knowledge about the regulation of Th cell subset development in a BAI is important to understand the balance between protective and harmful Th cell responses.

The aim of this study was to investigate the possible immunomodulatory effect of biomaterials on *S. aureus*- and *S. epidermidis*-induced immune responses, by measuring human DC and T cell activation. Human DC activation and, subsequently, DC-mediated T cell proliferation and Th1/Th2 cell polarisation induced by *S. aureus* or *S. epidermidis* in presence of poly(D,L-lactic acid) (PDLLA) and poly(trimethylene carbonate) (PTMC) were studied. PDLLA and PTMC are biodegradable and biocompatible materials provoking a normal foreign body response, as commonly seen after implantation of sterile biodegradable polymers (Pego *et al.*, 2003; Schakenraad *et al.*, 1989). PTMC may be slightly more immunoreactive, since it supports good immune cell adhesion (Bat *et al.*, 2009; Shokouhi *et al.*, 2010). Our results showed that both PTMC and PDLLA influenced DC cytokine production in response to *S. aureus* and *S. epidermidis*, but that neither polymer influenced the *staphylococcus*-induced T cell proliferation nor T cell polarisation. In the absence of the staphylococci, neither biomaterial induced DC-mediated T cell activation or polarisation, indicating that these materials are not immune-activating on their own in our human cell model. Furthermore, it was shown that *S. aureus* bacteria were stronger inducers of DC cytokine secretion, T cell proliferation and Th1 cell development than *S. epidermidis*, either in absence or in presence of the biomaterials.

## Materials and Methods

### Preparation of biomaterial films

Biodegradable polymer films were fabricated using a compression moulding machine (Fonteijne THB008, Vlaardingen, the Netherlands). Medical grade PDLLA (inherent viscosity 0.2 dL/g, used without further purification; Purac Biochem, Gorkum, the Netherlands) was moulded using a 200 μm-thick stainless-steel mould at approximately 150 °C and 250 kN/cm$^2$ load-pressure. Medical grade PTMC (molecular weight 250 kg/mol, used without further purification; Foryou Medical, P.R., Huizhou, China) was moulded using a 500 μm-thick stainless-steel mould at approximately 160 °C and 350 kN/cm$^2$ load-pressure. The PTMC film was exposed to 25 kGy gamma irradiation from a $^{60}$Co source (Synergy Health, Ede, the Netherlands) in a nitrogen atmosphere in order to crosslink the polymer. Scanning electron microscopy imaging showed that the surfaces of PDLLA and PTMC films had a smooth appearance without pores (data not shown). Disks with a diameter of 15 mm, to fit in the wells of 24-wells cell culture plates, were punched out of the PDLLA and PTMC films using a hole punch. The disks were disinfected with 70 % aqueous isopropanol for 15 min followed by washing in sterile phosphate-buffered saline (PBS). The procedure was repeated three times. After the last wash, disks were immersed in sterile PBS and stored at room temperature until the experiment was performed on the next day.

### Bacteria

*S. aureus* strain ATCC 49230 and *S. epidermidis* strain RP62a (ATCC 35984; both from ATCC; Manassas, VA, USA), both biofilm-producing strains isolated from patients with a BAI (Beenken *et al.*, 2003; Christensen *et al.*, 1985), were used in the present study. Prior to each experiment, the bacteria were cultured at 37 °C under agitation in tryptic soy broth (TSB; BD Difco, Etten-Leur, the Netherlands) until the logarithmic growth phase. The viable bacteria were harvested by centrifugation and resuspended to the desired concentrations in Iscove's modified Dulbecco's medium (IMDM, Lonza, Walkersville, MD, USA) containing 10 % foetal calf serum (FCS, Thermo Fisher Scientific Inc., Logan, UT, USA).

### Generation and stimulation of DCs

Immature DCs (iDCs) were generated and cultured as previously described (de Jong *et al.*, 2002; Sallusto and Lanzavecchia, 1994). In brief, monocytes were isolated from heparinised blood of healthy human donors by density gradient centrifugation on Lymphoprep™ (Nycomed, Oslo, Norway) and Percoll® (GE Healthcare, Uppsala, Finland) density gradient media. Isolated monocytes ($4 \times 10^5$ cells/well) were cultured in 24-well cell culture plates in IMDM (Gibco, Grand Island, NE, USA) supplemented with 5 % FCS, 86 μg/mL gentamicin (Duchefa, Biochemie B.V., Haarlem, the Netherlands), 500 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Whitehouse Station, NJ, USA) and 10 IU/mL recombinant human IL-4 (Miltenyi Biotec, Bergisch Gladbach, Germany). After 3 d, half of the cell culture medium was replaced with fresh medium. After 6 d, monocytes were fully differentiated into iDCs, as confirmed by the loss of monocyte marker CD14. On day 6, iDCs were resuspended (approximately $1 \times 10^5$ cells/well) and stimulated for 48 h with *S. aureus* or *S. epidermidis* bacteria [$1 \times 10^7$ colony forming unit (CFU)/well] in the absence or presence of PDLLA or PTMC discs. To prevent bacterial overgrowth, 10 μg/mL moxifloxacin (Avelox, Bayer Schering Pharma, Mijdrecht, the Netherlands) was added to all conditions tested. The ratio between iDCs and staphylococci (1 : 100) used in these co-culture experiments was based on the ratio leading to a level of DC maturation equal to that of DCs stimulated with 100 ng/mL lipopolysaccharide (from *Escherichia coli* 0111:B4) or 20 μg/mL Poly(I:C) (both purchased from Sigma-Aldrich, St. Louis, CA, USA). No major differences in DC viability nor in the proportion of apoptotic cells were observed after stimulation, as examined by flow cytometry using propidium iodide (Sigma-Aldrich) and Annexin V (BD Biosciences, San Jose, CA, USA) staining, respectively.

### Isolation of autologous naive T cells

To investigate the primary response of T cells, naive CD4$^+$ T cells were used. Naive T cells were obtained by isolating PBMCs from the blood of healthy human donors by density gradient centrifugation on Lymphoprep™ and Percoll® density gradient media and finally by negative selection using the CD4$^+$ T cell isolation MACS kit (Miltenyi Biotec) followed by CD45RO-PE (Dako, Amstelveen, the Netherlands) and magnetic anti-PE beads (Miltenyi Biotech) (van der Aar *et al.*, 2011). The purity of the isolated naive CD4$^+$ (CD45RO$^-$CD45RA$^+$) T cells was > 97 %, as measured by flow cytometry (Canto II, BD Biosciences).

### DC maturation analysis

After 48 h of stimulation, DC maturation was analysed by measuring the secreted cytokines in the supernatants and the expression of cell surface molecules. Supernatants were harvested and stored at −20 °C until the levels of the cytokines TNF-α (eBioscience, Logan, UT, USA), IL-10 (BD Pharmingen), IL-6 (UCytech, Utrecht, the Netherlands), IL-23 (UCytech), IL-1β (Endogen, Woburn, MA, USA) and IL-12p70 (UCytech) were analysed by sandwich enzyme-linked immunosorbent assays (ELISAs) (de Jong *et al.*, 2002). The expression of cell surface molecules was analysed by flow cytometry (Canto II, BD Biosciences) after staining with fluorescent antibodies against HLA-DR, CD83 and CD86 (all purchased from BD Biosciences). For each fluorescent

antibody, cells stained with a single fluorophore were used to correct for false positive fluorescence (data not shown). A gate, based on forward and side scatters, was set to exclude dead cells, cell debris and doublets.

**T cell proliferation**

To study autologous naive $CD4^+$ T cell proliferation, DCs were stimulated for 48 h as described above, washed and co-cultured for 5 d with carboxyfluorescein succinimidyl ester- (CFSE; 0.5 μm, Life Technologies, Eugene, OR, USA) labelled autologous naive $CD4^+$ T cells ($CD45RO^-CD45RA^+$) at a ratio of 1 : 1 ($4 \times 10^4 : 4 \times 10^4$ cells). T cell proliferation was determined by flow cytometry (Canto II, BD Biosciences) and quantified using FlowJo software (version 7.6.5, Tree star, Ashland, OR, USA). The division and proliferation indices were calculated. The division index indicates the average number of cell divisions for all cells in the original culture (non-dividing cells included); the proliferation index represents the average number of divisions of the responding population (non-dividing cells excluded). By definition, the value of the proliferation index is ≥ 1.0.

**Th cell polarisation**

In order to study the polarisation of autologous naive $CD4^+$ T cells, $1 \times 10^5$ iDCs, $1 \times 10^7$ CFU of *S. aureus* or *S. epidermidis* and $1 \times 10^5$ T cells were co-cultured in cell culture medium in absence or presence of the biomaterials. After approximately 4 d, half of the medium was replaced with T cell culture medium consisting of IMDM (Lonza) with 10 % FCS, 86 μg/mL gentamicin and 20 U/mL recombinant human IL-2 (Novartis AG, Nuremberg, Germany) to promote T cell survival and proliferation. Every 2 d, half of the medium was replaced with fresh T cell culture medium and cell suspensions were diluted, if necessary, until T cell cultures were resting (approximately by day 13). Resting $CD4^+$ T cells were re-stimulated for 5 h with 100 ng/mL phorbol myristate acetate (PMA), 1 μg/mL ionomycin and 10 μg/mL brefeldin A (BFA) (all purchased from Sigma-Aldrich) to allow detection of intracellular production of the Th1 and Th2 cell signature cytokines IFN-γ and IL-4, respectively. $CD4^+$ T cells were analysed by flow cytometry after fixation with 3.7 % formaldehyde (Sigma-Aldrich) and permeabilisation with 0.5 % saponin (Calbiochem, Darmstadt, Germany) followed by intracellular staining with anti-IL-4 and anti-IFN-γ (both from BD Bioscience) antibodies for 30 min (de Jong *et al.*, 2002). For both fluorescent antibodies, cells stained with a single fluorophore were used to allow for proper gating of IFN-γ- and IL-4-positive cells (data not shown).

**Statistical analysis**

The data were analysed using linear mixed models (LMM) (covariance type: compound symmetry) on rank-transformed data with donor as covariate, followed by the *post-hoc* Wilcoxon signed ranked test for pairwise comparisons. The immunomodulatory effect of the combination of biomaterials and staphylococci was measured by assessing markers related to four different outcome groups: DC maturation marker expression, DC cytokine production, Th cell polarisation and T cell proliferation. The linear mixed model was considered to be statistically significant at $p \leq 0.0125$ (adjusted for four outcome groups). The Wilcoxon signed ranked test was considered to be statistically significant at $p \leq 0.05$. Missing values in our data set resulted in unequal number of donors per tested condition. The LMM produces valid estimates even in the case of missing values. Analysis of the more restricted data set only using donors with a complete set of conditions confirmed the validity of our approach since the statistical interpretation of the results remained the same as compared to the results using the total data set with all donors. Statistical analyses were performed using IBM SPSS Statistics software version 24 (IBM Corp., Armonk, NY, USA).

## Results

**Minimal influence of PDLLA and PTMC on *Staphylococcus*-induced DC maturation marker expression**

DC maturation, characterised by an increase in the expression of the co-stimulatory molecules CD86 and CD83 and of HLA-DR, is central to the induction of adaptive T cell responses. Exposure of DCs to PDLLA or PTMC did not affect these markers, whereas DCs exposed to *S. aureus* or *S. epidermidis* expressed significantly higher levels of the co-stimulatory molecules and HLA-DR than unstimulated DCs (Fig. 1**a**,**b**). When DCs were exposed to biomaterials and bacteria combined, the presence of PDLLA did not affect the *S. aureus*- or *S. epidermidis*-induced DC marker expression (Fig. 1**a**,**c**). The presence of PTMC caused a small but significant reduction of the *S. epidermidis*-induced CD86 expression (Fig. 1**a**,**c**). Interestingly, exposure to the combination of PTMC and staphylococci caused slightly lower levels of CD86 expression as compared with the combination of PDLLA and staphylococci (Fig. 1**a**), indicating that the two biomaterials differentially affected CD86 expression in presence of staphylococci. Taken together, PDLLA and PTMC had no, or only minor, effects on *S. aureus*- and *S. epidermidis*-induced DC maturation marker expression.

**DC cytokine secretion in response to the combination of staphylococci and biomaterials**

In order to assess the potential of the DCs to polarise Th cells, DC cytokine production was studied. IL-1β was undetectable in all cases (data not shown). In incubations with only biomaterials, DCs exposed to PDLLA or PTMC did not produce detectable levels of

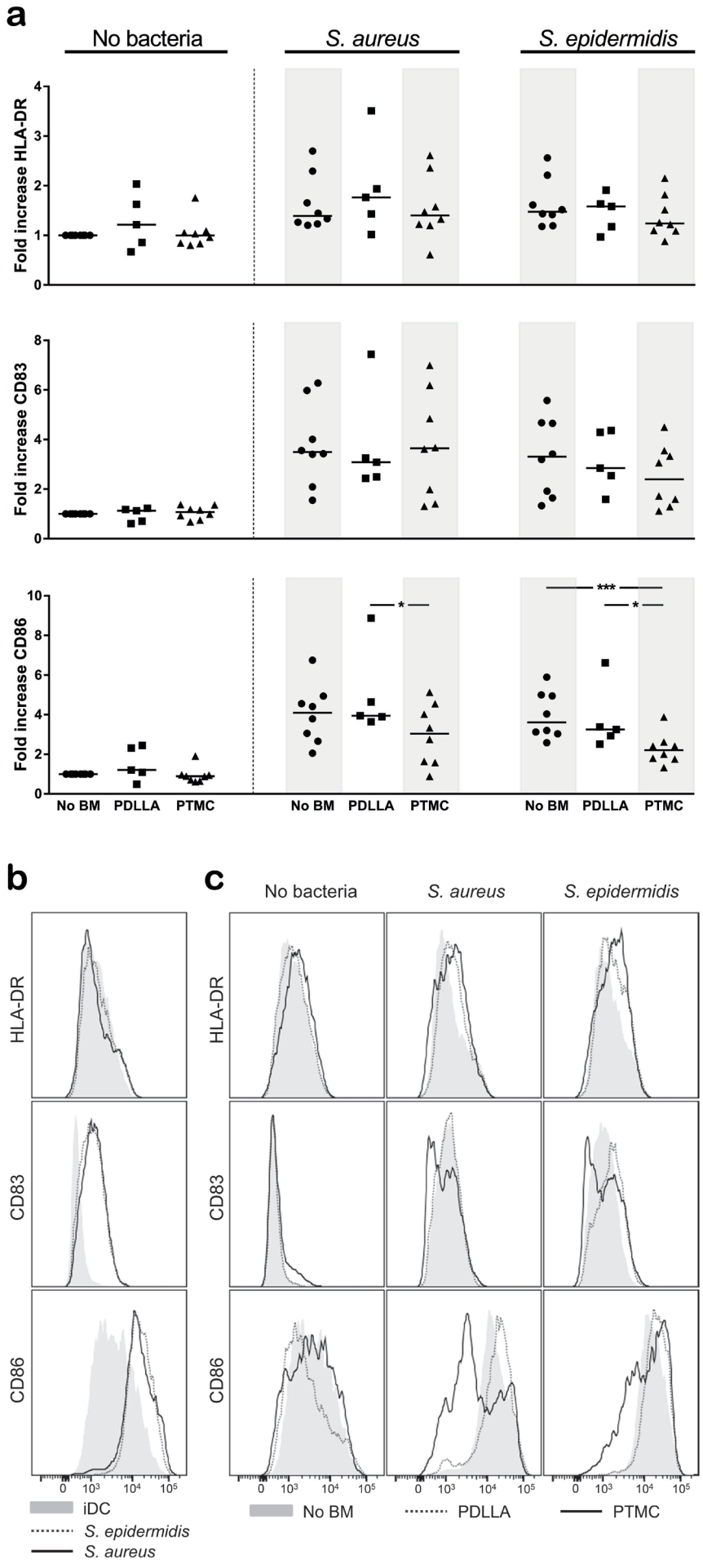


**Fig. 1.** Combined effect of biomaterials and staphylococci on DC maturation marker expression. (**a**) Fold difference of geometric mean fluorescence intensity of HLA-DR, CD83 and CD86 of stimulated relative to unstimulated DCs; no bacteria and no biomaterial (BM). Each dot represents one donor. The horizontal line represents the median value per group. Exposure to *S. aureus* or *S. epidermidis* significantly enhanced the expression of all three DC maturation markers, when compared to unstimulated DCs (all $p \leq 0.01$, significance not indicated in the figure). * $p \leq 0.05$, *** $p \leq 0.001$. (**b**,**c**) Cell surface expression of HLA-DR, CD83 and CD86 ($log_{10}$ fluorescence intensity) on DCs. Data of 1 representative experiment out of 5-8 experiments with cells from different donors. (**b**) Expression on unstimulated (filled), *S. aureus*- (solid) or *S. epidermidis*- (dashed) stimulated DCs. (**c**) Expression on unstimulated DCs exposed to biomaterials in absence (left) or presence of *S. aureus* (middle) or *S. epidermidis* (right).

any of the cytokines analysed (Fig. 2). In incubations with only bacteria, DCs exposed to *S. aureus* secreted significantly higher levels of all tested cytokines than unstimulated DCs (Fig. 2). DCs exposed to *S. epidermidis* secreted low but significantly higher levels of TNF-α, IL-10, IL-6 and IL-23 than unstimulated DCs (Fig. 2). Interestingly, DCs exposed to *S. epidermidis* did not produce detectable levels of Th1-cell-polarising cytokine IL-12p70 (Fig. 2**e**), suggesting that these DCs would not drive Th1 cell development.

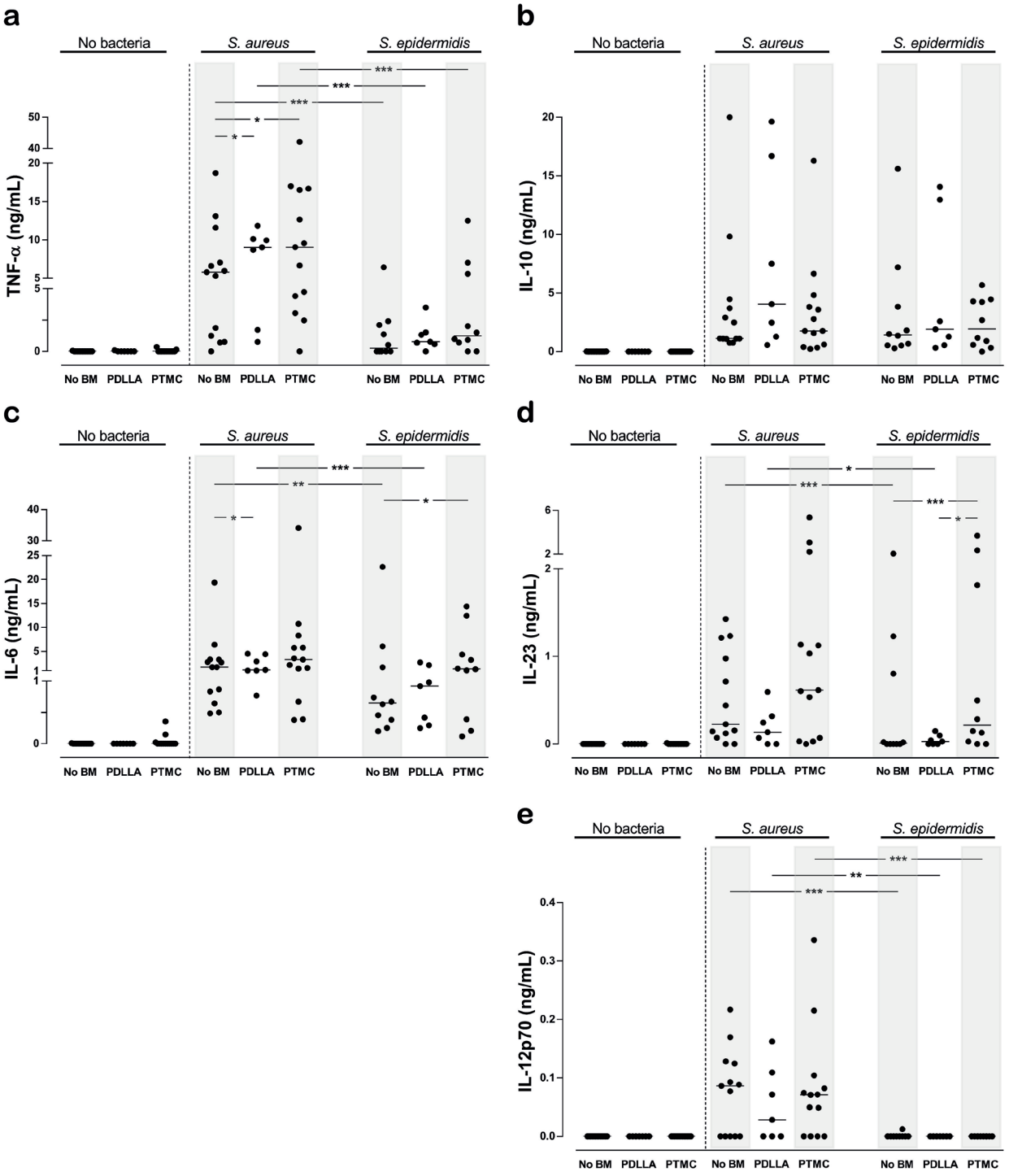


**Fig. 2.** (**a**-**e**) Production of cytokines by DCs upon exposure to staphylococci and/or biomaterials. DC cytokine concentrations determined by ELISA of 7-13 independent experiments with cells from different donors, performed in triplicate. Unstimulated DCs (no bacteria and no BM) and DCs exposed to PDLLA and PTMC did not produce detectable levels of any of the tested cytokines. Each dot represents the average cytokine concentration for a single donor. The horizontal line represents the median value per group. Compared to unstimulated DCs, DCs exposed to *S. aureus* and *S. epidermidis* induced higher levels of all tested cytokines (all $p \leq 0.001$, significance not indicated in the figure). (**a**) TNF-α; (**b**) IL-10; (**c**) IL-6; (**d**) IL-23; (**e**) IL-12p70. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

In combined exposure of DCs to biomaterials and bacteria, PDLLA slightly decreased the *S. aureus*-induced secretion of IL-6 and slightly enhanced the *S. aureus*-induced secretion of TNF-$\alpha$ (Fig. 2**a**,**c**). PTMC enhanced the *S. aureus*-induced secretion of TNF-$\alpha$ (Fig. 2**a**) and the *S. epidermidis*-induced secretion of IL-6 and IL-23 (Fig. 2**c**,**d**). Notably, the *staphylococcus*-induced secretion of IL-10 and IL-12p70 was not altered by presence of either of the biomaterials (Fig. 2**b**,**e**).

Of all cytokines tested, only IL-23 secretion was differently affected by PDLLA and PTMC in combination with *S. epidermidis* (Fig. 2**d**), indicating that differences in biomaterial characteristics minimally influenced the DC cytokine response. In contrast, *S. aureus* and *S. epidermidis* differently affected DC cytokine secretion. DCs exposed to *S. aureus* secreted higher levels of TNF-$\alpha$, IL-6, IL-23 and IL-12p70 than DCs exposed to *S. epidermidis* (Fig. 2). Moreover, in combination with PDLLA and

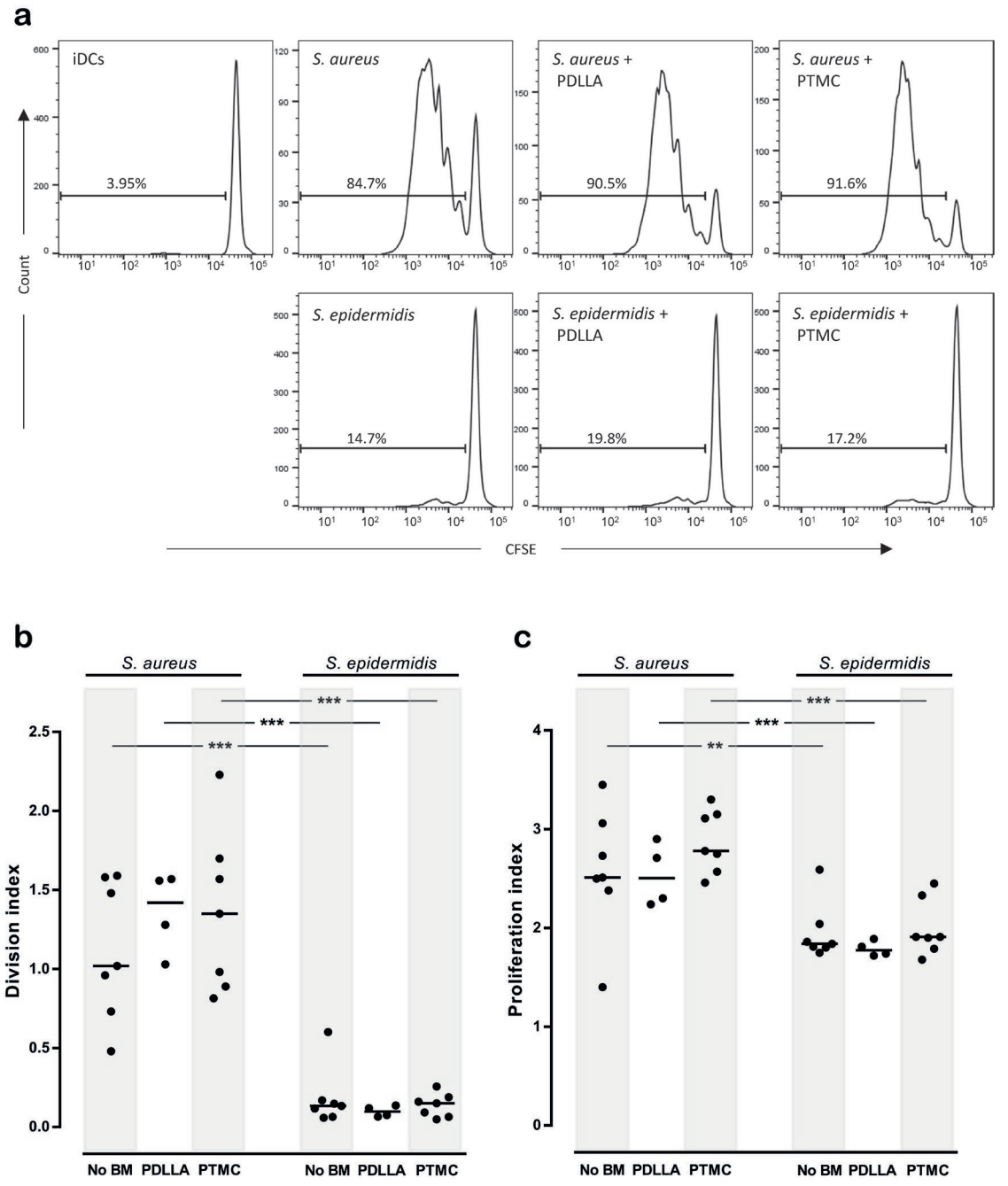


**Fig. 3.** Proliferation of antigen-specific naive CD4+ T cells after 5 d of co-culture with staphylococci- and/or biomaterial-exposed DCs. (**a**) CFSE profiles of T cells proliferation. Data of 1 representative experiment out of 4 or 7 experiments with cells from different donors ($log_{10}$ fluorescence intensity). The percentage of T cells proliferation is indicated. DCs exposed to only PDLLA or PTMC induced T cell proliferation similar to iDCs (data not shown). (**b**) Division index and (**c**) proliferation index per stimulus. Each dot represents one donor tested in an individual experiment. The horizontal line represents the median value. Data of 4 or 7 independent experiments. ** $p \leq 0.01$, *** $p \leq 0.001$.

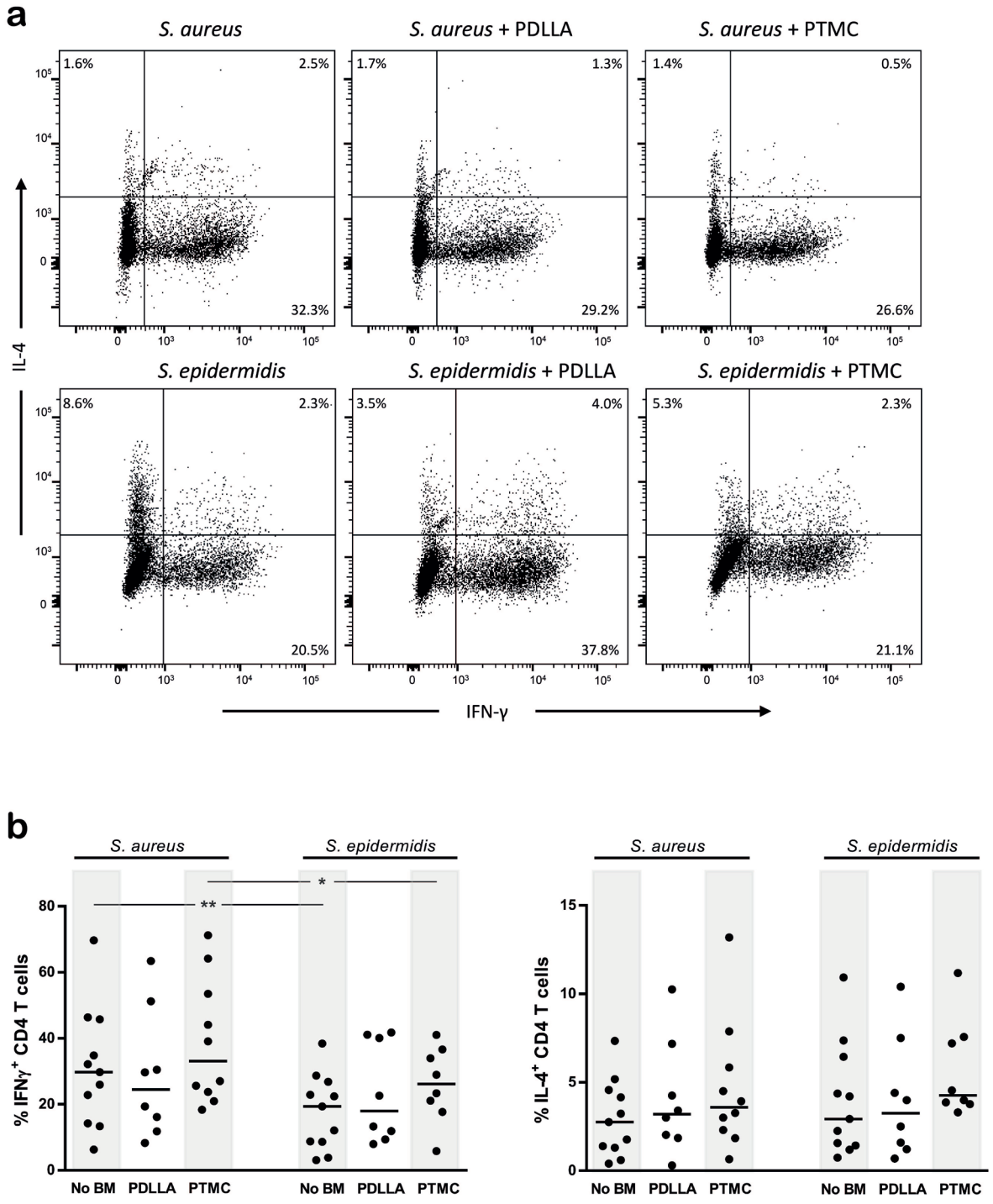


**Fig. 4.** Th1 and Th2 cell polarisation of antigen-specific naive CD4+ T cells induced by DCs exposed to staphylococci in combination with biomaterials. Th1 or Th2 cell polarisation of resting T cells was determined by measuring intracellular levels of IFN-γ and IL-4, respectively. (**a**) FACS dot plots of 1 representative experiment out of 8 or 11 experiments with cells from different donors. Intracellular expression of IFN-γ and IL-4 ($log_{10}$ fluorescence intensity). (**b**) Percentages of IFN-γ- or IL-4-expressing T cells. The horizontal line represents the median value. Data of 8 or 11 independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

PTMC, *S. aureus* induced higher levels of TNF-$\alpha$, IL-6, IL-23 and IL-12p70 secretion and of TNF-$\alpha$ and IL-12p70 secretion, respectively, compared with *S. epidermidis* (Fig. 2). Taken together, these results showed that simultaneous exposure to biomaterials and staphylococci slightly but significantly modified DC secretion of pro-inflammatory cytokines relative to the secretion induced by exposure to the bacteria alone. These changes in cytokine levels might affect DC-induced Th cell polarisation. Moreover, *S. aureus* bacteria proved to be more potent inducers of DC cytokine secretion than *S. epidermidis* bacteria.

**Antigen-specific T cell proliferation in response to staphylococci was not altered by PDLLA and PTMC**

Whether the DCs matured in presence of staphylococci and biomaterial were functionally active was determined by analysing their ability to induce antigen-specific naive $CD4^+$ T cell proliferation. DCs exposed to only PDLLA or PTMC did not support antigen-specific T cell proliferation (data not shown), similar to iDCs (Fig. 3**a**). DCs incubated with *S. aureus*, either in absence or presence of the biomaterials, were potent inducers of T cell proliferation, as judged based on the CFSE profiles of the T cells (Fig. 3**a**). In contrast, DCs exposed to *S. epidermidis*, either in absence or in presence of the biomaterials, induced only low levels of T cell proliferation (Fig. 3**a**). Analysis of the CFSE profiles indicated that the division index (the number of cell divisions of all T cells) after stimulation with *S. aureus* had a median value of 1.02 (Fig. 3**b**). Combined exposure to *S. aureus* and either PDLLA or PTMC did not significantly increase T cell proliferation, with division index values of 1.42 and 1.35, respectively (Fig. 3**b**). Exposure to *S. aureus* or *S. aureus* + PDLLA induced T cell proliferation with almost identical median proliferation index values of 2.51 and 2.50, respectively, reflecting the average number of divisions of the responding T cells (Fig. 3**c**). *S. aureus* in combination with PTMC induced T cell proliferation with a proliferation index value of 2.78, which was not significantly different from the value of T cells activated by *S. aureus* alone (Fig. 3**c**). The combination of *S. epidermidis* and PDLLA or PTMC caused a low level of T cell proliferation, with division index values of 0.10 and 0.15, respectively, and proliferation index values of 1.78 and 1.91, respectively. These values were not statistically different from *S. epidermidis*-induced division and proliferation index values (Fig. 3**b**,**c**). Our results indicated that the presence of the biomaterials did not influence the *S. aureus*- or *S. epidermidis*-induced T cell proliferation. Strikingly, *S. aureus* and *S. epidermidis* induced comparable expression of DC maturation markers, but DCs exposed to *S. aureus* induced significantly more T cell proliferation than *S. epidermidis*-exposed DCs, in absence as well as in presence of the biomaterials (Fig. 3**b**,**c**).

Together, these data indicated that PDLLA and PTMC did not influence *Staphylococcus*-induced T cell proliferation, but a clear difference was observed between *S. aureus*- and *S. epidermidis*-induced T cell proliferation.

**PDLLA and PTMC did not alter *Staphylococcus*-induced Th1 or Th2 cell polarisation**

The combination of *S. aureus* or *S. epidermidis* with the biomaterials influenced the production of different levels of TNF-$\alpha$, IL-6 and IL-23 from DCs (Fig. 2), suggesting that Th cell polarisation directed by these DCs might be different as well. To determine Th cell polarisation, intracellular levels of IFN-$\gamma$ and IL-4, as markers for Th1 and Th2 cells, respectively, were analysed. Since unstimulated DCs (iDCs) and DCs exposed to PDLLA and PTMC did not induce Th cell proliferation, no polarisation could be recorded. In accordance with earlier studies (van Beelen *et al.*, 2007), *S. aureus* predominantly induced Th1 cells from naive T cell precursors and only low levels of Th2 cells (Fig. 4**a**,**b**). DCs exposed to *S. epidermidis* secreted undetectable levels of IL-12p70 (Fig. 2**e**) and induced low levels of T cell proliferation (Fig. 3). The T cells, which proliferated upon *S. epidermidis* exposure, were predominantly Th1 cells. Combining *S. aureus* or *S. epidermidis* with either of the biomaterials did not significantly change the Th1/Th2 polarisation relative to the effect of *S. aureus* or *S. epidermidis* alone (Fig. 4**b**). In line with the higher induction of DC cytokines, *S. aureus* was also a more potent inducer of Th1 cell development than *S. epidermidis* (Fig. 4**b**) Collectively, these data indicated that neither of the biomaterials affected *S. aureus*- or *S. epidermidis*-induced Th1 and Th2 cell polarisation, but the two bacterial species did differ in their potency to induce Th1 cell development.

## Discussion

The combined presence of a biomaterial and bacteria may cause derangements of local immune responses, leading to a BAI. Little is known about the regulation of human $CD4^+$ Th cells in BAIs, while inadequate Th cell activation and regulation by DCs may be responsible for inadequate cellular responses against bacteria causing BAIs. In this study, an *in vitro* human cell system was used to study DC and T cell responses to *S. aureus* and *S. epidermidis*, major causes of BAIs, in the absence and presence of PDLLA and PTMC. It was shown that PDLLA and PTMC had minor effects on immune responses of DCs and T cells to the staphylococci. Although PTMC slightly decreased and, both, PDLLA and PTMC slightly modified *S. aureus*- and *S. epidermidis*-induced DC marker expression and DC pro-inflammatory cytokine production, respectively, these changes in the DC responses did not affect the DC-induced T cell proliferation or Th1/Th2 cell polarisation. Moreover, *S. aureus* was found to be a more potent inducer of DC cytokine secretion, T cell proliferation and Th1 cell development than *S. epidermidis*. Together,

these observations indicated that PDLLA and PTMC slightly influenced *S. aureus*- and *S. epidermidis*-induced DC responses, but that this did not lead to changes in Th cell development.

Polymers such as poly(lactic-co-glycolic acid) (PLGA) and a copolymer of PTMC and poly(L-lactic acid) (LT706 or PLTMC) enhance DC-mediated T cell proliferation or even modulate Th cell polarisation in response to model antigens (Petrizzo *et al.*, 2015; Shokouhi *et al.*, 2010; Yoshida and Babensee, 2004). These immunomodulatory properties of biomaterials are of great interest in shaping the immune response in diverse fields, such as cancer vaccine development, treatments for allergies and treatments for autoimmune diseases. On the other hand, modulation of Th cell responses by biomaterials, resulting in reduced bacterial clearance, can be deleterious. The results of this study showed that neither PDLLA nor PTMC affected the *S. aureus*- or *S. epidermidis*-induced T cell proliferation or Th1/Th2 cell development. This suggested that in this human cell model and with these biomaterials, T cell responses were mainly determined by the bacterial species.

In a BAI, staphylococci can reside on the biomaterials in biofilms and they can survive inside or outside macrophages in the tissue surrounding the biomaterial, due to reduced efficacy of the local immune response against the bacteria (Busscher *et al.*, 2012; Zaat *et al.*, 2010). Protection against intracellular survival of staphylococci requires Th1 cell responses. In addition, Th17 cells play an important role in protective immunity against staphylococci (Brown *et al.*, 2015; Milner *et al.*, 2008). In the present study, human naive CD4+ T cells were used to investigate the primary Th cell response to the combination of staphylococci and biomaterials. It was found that *S. aureus*- and *S. epidermidis*-activated DCs promoted Th1 and Th2 cell development, but they were not capable of promoting Th17 cell development from naive $CD4^+$ T cells *in vitro*, neither in absence nor in presence of PDLLA or PTMC (data not shown). The requirements for induction of antigen-specific Th17 cell development from truly naive $CD4^+$ T cells by antigen-presenting cells (APCs) in humans are still under debate, but APCs do promote Th17 cell development from human memory $CD4^+$ T cells (Evans *et al.*, 2007; van Beelen *et al.*, 2007). Since such cells were not used in the current work, this may explain the absence of a Th17 response. However, DCs exposed to the combination of *S. epidermidis* and PTMC produced larger amounts of the Th17 cell-polarising cytokines IL-6 and IL-23 than DCs exposed to *S. epidermidis* alone. This might indicate the potential of these DCs to induce a Th17 cell response, which might have been observed if memory T cells were included (van Beelen *et al.*, 2007).

Exposure of murine and human DCs to several polymeric biomaterials induces upregulation of DC maturation markers (Babensee and Paranjpe, 2005; Shokouhi *et al.*, 2010). PLTMC, a copolymer containing trimethylene carbonate (TMC) monomers, induces maturation marker expression in murine DCs *in vitro* (Shokouhi *et al.*, 2010). In contrast, our data showed that exposure of human iDCs to PTMC did not induce upregulation of maturation markers. This difference may be related to the use of DCs from distinct species, since the intensity of the murine DC maturation marker expression in response to a biomaterial may be different from that of human DCs (Yoshida and Babensee, 2004; Yoshida *et al.*, 2007), or to differences in physicochemical properties between PLTMC and PTMC. Although PTMC alone did not affect DC maturation marker expression, PTMC slightly decreased the CD86 expression induced by *S. epidermidis* (Fig. 1), while T cell activation was not changed. In general, low expression of co-stimulatory molecules is associated with low T cell activation or with the induction of regulatory T cells (Nurieva *et al.*, 2010). The lower CD86 expression induced by the combination of PTMC and *S. epidermidis* was still significantly higher when compared to the expression of these molecules by iDCs. Indeed, despite the lower level of co-stimulatory molecule expression, the stimulated DCs were capable of activating T cells.

Sensing of biomaterials by DCs is thought to be mediated by DC pattern recognition receptors, such as toll-like receptors (TLRs) and integrins (Rogers and Babensee, 2011; Shokouhi *et al.*, 2010). These receptors may recognise structures in the layer of host proteins, which absorb to the biomaterial surface upon implantation, or may sense the biomaterial surface directly (Rogers and Babensee, 2011; Shokouhi *et al.*, 2010). Variations in the absorbed protein layer depending on biomaterial chemical characteristics such as hydrophobicity, will influence recognition by DC receptors and subsequent DC activation (Shankar *et al.*, 2010). PDLLA and PTMC are both hydrophobic biodegradable polyesters, but PTMC is more flexible, a characteristic which may enhance cell attachment and activation (Evans *et al.*, 2009; Li *et al.*, 2014). Moreover, the degradation products of PDLLA and PTMC may also have different effect on cells. The degradation products of PDLLA are acidic, while the degradation products of PTMC are not (Lu *et al.*, 1999; Zhang *et al.*, 2006). Acidic degradation products can be detrimental to cells and can lead to inflammatory reactions (Yi *et al.*, 2016). In our study, PDLLA and PTMC did not differently affect DC responses in the absence of staphylococci. However, in the presence of staphylococci, these biomaterials did slightly differ in their induction of CD86 expression and IL-23 secretion by DCs. This might indicate that differences in immunomodulating potential of biomaterials might only become evident in the presence of strong immune-stimulators, such as bacteria. Biomaterials might modulate DC responses to bacteria by enabling cross-talk of different DC receptors simultaneously recognising the biomaterial and bacteria. Cross-talk between multiple DC receptors can result in altered

DC activation (den Dunnen *et al.*, 2012; Mitchell *et al.*, 2010; Re and Strominger, 2004; van Kooyk and Geijtenbeek, 2003). Simultaneous ligation of Fc-gamma-receptor-IIa (FcγRIIa) and TLR2, 4 or 5 induces a synergistic cytokine response by various APCs (den Dunnen *et al.*, 2012; Vogelpoel *et al.*, 2015), while FcγRIIa ligation on its own does not lead to a cytokine response. The observation that either PTMC or staphylococci as a single stimulus did not, or only moderately, induce DC cytokine secretion, whereas the combination of both stimuli induced increased levels of cytokines, might suggest a possible similar crosstalk between TLRs recognising bacterial cell envelope components and/or biomaterial structures and FcγRIIa recognising antibody-opsonised staphylococci and antibodies absorbed to the PTMC surface. However, blocking DC FcγRIIa did not prevent the increased cytokine production by the DCs stimulated by staphylococci combined with PTMC (data not shown). This result indicated that cross-talk with FcγRIIa had no role in the increased DC cytokine response observed. Other DC receptors, such as C-type lectins or integrins may be responsible for the modulation of inflammatory cytokine responses.

The increased DCs pro-inflammatory cytokine response to staphylococci in the presence of PTMC and PDLLA may point to non-Th-cell-mediated modulation of the local immune response at the site of infection. Upon recognition of bacteria, DCs, not only initiate adaptive T cell responses, but also create a local cytokine environment, which attracts and affects other innate immune cells (Lewis and Reizis, 2012). DCs, which stimulate a very strong pro-inflammatory environment, may hyperactivate other phagocytic immune cells, such as monocytes and macrophages; thereby, compromising their intracellular bactericidal function, allowing ingested bacteria to survive (Kanangat *et al.*, 1999). To elucidate multicellular immune responses in BAIs, *in vivo* animal models or *ex vivo* human models could be used.

Interestingly, staphylococci-induced DC and T cell responses were not uniform between *S. aureus* and *S. epidermidis*, regardless of the presence of PDLLA or PTMC. *S. aureus*-stimulated DCs secreted moderately higher levels of pro-inflammatory cytokines than *S. epidermidis*-stimulated DCs. Moreover, *S. aureus* was a strong inducer of T cell proliferation and Th1 cell development in contrast to *S. epidermidis*. Consistent with our results, *S. aureus* is shown to be a more potent inducer of IFN and IL-12p70 production by human mononuclear cells than *S. epidermidis* (Buzas *et al.*, 2004). The mechanism underlying the enhanced DC cytokine response to *S. aureus* is still to be elucidated, but it may be related to the multiple virulence factors expressed by *S. aureus*, such as staphylococcal enterotoxins (Coutant *et al.*, 1999; Stuyt *et al.*, 2001). In addition, staphylococcal enterotoxins likely also play a role in the high T cell proliferation observed in response to *S. aureus*. These enterotoxins can bypass the need for antigen processing and presentation by directly cross-linking MHC-II molecules on APCs with T cell receptors, triggering activation/proliferation of large numbers of T cells in an antigen-independent fashion (Spaulding *et al.*, 2013). This strong immune-activating potential of *S. aureus* correlates with more aggressive progression of *S. aureus* infections as compared with *S. epidermidis* infections (Subbiahdoss *et al.*, 2011). The differences in intensity of DC and T cell activation between *S. aureus* and *S. epidermidis* may, apart from biofilm formation, be an important factor in the pathogenesis of BAIs caused by these bacteria.

## Conclusion

Taken together, our data indicated that PTMC and PDLLA did not interfere with the DC-mediated Th1 and Th2 cell response to *S. aureus* and *S. epidermidis*. The slightly modified DC inflammatory response to staphylococci in presence of PTMC and PDLLA did not lead to differences in T cell responses. However, these slightly altered DC responses might affect bacterial clearance in a multicellular setting, *e.g.* including phagocytic cells such as macrophages. In summary, in designing new biomaterials, it is important to take into account that biomaterials can influence the normal immune response to bacteria and a human cell system, such as the one that was used in this study, is a valuable tool to discern such influences on the human immune response.

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**Editor's note**: All of the matters raised by the reviewers have been incorporated in the main text, so there is no Discussion with Reviewers section for this paper.

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